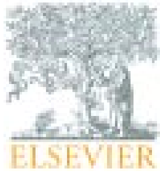

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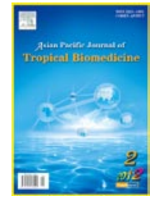

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Validated HPTLC analysis method for quantification of variability in content of curcumin in *Curcuma longa* L (turmeric) collected from different geographical region of India

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ABSTRACT

Objective: To develop a simple, sensitive, precise, and accurate stability-indicating high performance thin-layer chromatographic method for analysis of curcumin (the main active constituent of turmeric). **Methods:** The separation was achieved on TLC aluminum plates precoated with silica gel $60F_{254}$ using toluene-chloroform-methanol (5:4:1, v/v/v) as a mobile phase. Densitometric analysis was performed at 430 nm. **Results:** This system was found to have compact spot of curcumin at RF value of (0.31±0.02). For the proposed procedure, linearity (r2 = 0.99354 ± 0.00120), limit of detection (50 ng/spot), limit of quantification (200 ng/spot), recovery (ranging from 98.35% - 100.68%), and precision (⩽2.25%) were found to be satisfactory. Statistical analysis reveals that the content of curcumin in different geographical region varied significantly. **Conclusions:** The highest and lowest concentration of curcumin in Turmeric was found to be present in sample of Erode (Tamilnadu) and Surat (Gujrat) respectively which inferred that the variety of turmeric found in Erode (Tamilnadu) is much superior to other region of India.

1. Introduction

Curcumin 1, 7-bis-(4-hydroxy-3-methoxyphenyl)-1, 6- heptadiene-2, 5-dione (Figure 1) is a yellow colored phenolic pigment obtained from powdered rhizome of *Curcuma longa* Linn. (Family: Zinziberaceae) (*C. longa*). Curcumin is the main active constituent of *C. longa* L [1] .This molecule has a broad range of activities including antioxidant, anti inflammatory, anticarciogenic, hypocholesterolemic, wouldhealing, antispasmodic, anticoagulant, antitumor and hepatoprotective activities[2]. *C. longa* L. commonly known as turmeric (Haldi) is a well-known plant which is used as a drug in Ayurvedic and Unani system of medicine [3, 4]. It is a pan tropical crop cultivated widely in South East Asia. It has a wide range of biological activities including anti-inflammatory[5-7] anti-Alzheimer's disease, [8-10] anti-tumorigenesis, [11-13] anti-angiogenesis, [14, 15] anti-diabetes [16-18]. Turmeric contains the main active constituent curcumin, and other constituents including demethoxycurcumin, bisdemethoxycurcumin (4-6%), essential oil, (2-4%) fixed oil (2-3%) and various volatile oils, including turmerone, atlantone, and zingiberone. It also contains sugars, proteins and resins [19] . Turmeric comprises about 70 species and highest diversity is concentrated in India (40 species). India is the largest producer, consumer and exporter of Turmeric in the world [20]. Hence it is imperative to know the geographical region of India which contains the highest quantity of main active constituent (curcumin) in turmeric.

In recent years several analytical techniques have been established for the qualitative and quantitative analysis of Curcuma species including GC-MS, [21] and HPLC [22-24]. A chromatographic comparison between HPLC and HPTLC method has been reported for quality control of curcumin but very few studies have been reported to determine curcuminoids in different Curcuma species or in the same species of samples collected from different cultivation regions and this information is critically important for the quality control of related herbal medicines [25] . Hence, it

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would be very significant to develop an efficient analytical method that can analyze curcumin of *C. longa* collected from different geographical regions. However, to our knowledge, no high-performance thin-layer chromatographic (HPTLC) method for determination of curcumin in *C. longa* collected from different geographical region of India has been ever reported.

2. Experimental

2.1. Plant material

Rhizomes of *C. longa* were collected from different parts of India .The plant specimens (32, 109, 5, 77, 9, 451, 21, and 67) were authenticated by taxonomist Prof. M. P. Sharma, Dept. of Botany, Jamia Hamdard University, New Delhi (India).

2.2. Chemicals and reagents

All reagents used in the development were purchased from Merck, India. Analytical standard of curcumin (Assigned purity: 98%) was obtained from Ms. Chroma Dex, Santa Ana, CA, USA. The identity of curcumin was confirmed by comparing their spectral data with those previously reported[26] .

2.3. Extraction of plant material for analysis

Eight different specimens of *C. longa* rhizomes (each of 1 g air dried fresh rhizomes) procured from various locations like (south, west, east and north) of India were extracted by reflux using 50 mL of HPLC grade methanol for minimum of 2 hours at temperature 70℃ and then filtered through Whatman No# 42. Extracts obtained after the reflux were concentrated on hot water bath (HH-6 Digital thermostatic, Jintan medical instrument) and finally the volume was adjusted to 50 mL with methanol for HPTLC analysis.

2.4. TLC instrumentation & conditions

The chromatography was performed for extracts as per method reported earlier from our lab [27]. In brief, the samples were spotted in the form of bands of width 3 mm with a Camag micro litre syringe on precoated silica gel aluminium plate $60F_{254}$ (20 cm×10 cm with 0.2 mm thickness; E. Merck, Darmstad, Germany) using a Camag Linomat V (CAMAG, Muttenz, Switzerland). A constant application rate of 150 nL/s was employed and space between two bands was 4 mm. The slit dimension was kept at 4 mm×0.1 mm, and 20 mm/s scanning speed was employed. These parameters were kept constant throughout the analysis of samples. The mobile phase consisted of toluene, chloroform and methanol in a ratio of 5: 4: 1 v/v/v.

Plates were developed in ascending order with a CAMAG twin trough glass tank which was pre-saturated with the mobile phase for 15 min; the length of each run was 8 cm. The TLC runs were performed under laboratory conditions (Temp: 25 ± 2 ℃ and % RH: 60 ± 5). The plates were then dried in air. Densitometric analysis was performed at 430 nm with a Camag TLC scanner III operated by Win CATS software (Version 1.2.0). The source of radiation utilized was deuterium and tungsten lamp.

The composition of the mobile phase for TLC was optimized using different solvents of varying polarity and good resolution was achieved using toluene: chloroform: methanol (5: 4: 1, v/v/v) as mobile phase. The RF value for Curcumin was found to be 0.31±0.02. The scanning wavelength selected was 430 nm, the absorption maxima of the curcumin spot.

2.5. Calibration curve of curcumin

A stock solution of curcumin (500 μg/mL) was prepared in methanol. Dilutions were made in methanol as 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1, 2 and 4 μL from the stock and were spotted on TLC plate in six (n=6) to obtain final concentration range of 50-1000 ng/spot. The data of peak area versus drug concentration were treated by linear least-square regression. QC samples chosen for the study were 200, 400 and 800 ng/pot.

2.6. Method validation

The developed method is validated as per the ICH guidelines. Method validation is carried out to confirm that the analytical method employed for this specific analysis is suitable for its intended use. Results from method validation can be used to check its quality, reliability and consistency. The method was validated by determining linearity, precision, accuracy, limits of detection (LOD), limits of quantification (LOQ), and recovery.

2.6.1. Precision and accuracy

The intra-day precision and accuracy of the assays were evaluated by performing replicate analyses (n = 6) of QC samples (200, 400 and 800 ng/spot). The inter-day precision and accuracy of the assay was determined by repeating the intra-day assay on three different days. Precision was expressed as the percentage coefficient variation (CV,%) of measured concentrations for each calibration level, whereas accuracy was expressed as percent recovery [28] .

2.6.2. Sensitivity and linearity

In order to estimate detection (LOD) and quantification (LOQ) limits, we spotted blank methanol (n = 6) following the same method as explained under the section of chromatographic conditions and the standard deviation (σ) of the magnitude of analytical response was determined. The LOD was expressed as (LOD = 3.3σ/slope of curcumin calibration curve), whereas LOQ was expressed as (LOQ = 10σ/slope of curcumin calibration curve) [29] .

2.6.3. Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for curcumin in sample was confirmed by comparing RF and spectra of spot with that of standard. The peak purity of curcumin was assessed by comparing the spectra at three different levels i.e. peak

start, peak apex and peak end positions of the spot[29].

3. Results

3.1. Selection and optimization of mobile phase

Initially Toulene: Choloroform: Methanol in varying ratios was investigated. The mobile phase Toulene: Choloroform: Methanol (5:4.5:0.5, v/v/v) gave good resolution with RF value of 0.31 for curcumin but typical peak nature was missing. The volume ratios of chloroform of over said system was varied to determine the effect on RF and on the response to curcumin. Finally, the mobile phase consisting of Toluene: Choloroform: Methanol (5:4:1, v/v/v) gave a sharp and well-defined peak at RF value of 0.31 (Figure 2). Well-defined spots were obtained when the chamber was saturated with the mobile phase for 15 min at room temperature.

For determination of the linearity curves of area vs concentration, different amounts of stock solution of curcumin was applied on the HPTLC plate and analysed.

Table 1

Linear regression data for the Calibration plot (n=6).

Parameters	Observation
Linearity range (ng/spot)	200-1000
Correlation coefficient (r2)	0.99354
Regression equation	Y=6597.142+ 13.240X
Slope± SD	13.240± 0.372
Interceptb± SD	6597.142 ± 0.984
LOD (ng/spot) (Limit of detection)	50
LOQ (ng/spot) (Limit of quantification)	200

Y= Peak area, X=Concentration (ng / spot)

3.2. Calibration curve

Calibration was linear in the concentration range 200-1000 ng. The linear regression equation was Y = 6597.142+ 13.240 X, for curcumin, while the correlation coefficients (r2) was 0.99354, with high reproducibility and accuracy (Table 1).

Detection limit of curcumin was determined by plotting a series of concentrations on the plate and scanning at 430 nm. The lowest amount of curcumin, which could be detected (LOD), was 50 ng/spot. The lowest amount of curcumin which could be quantified (LOQ), was found to be 200 ng/spot.

3.3. Validation of method

3.3.1. Recovery studies

The proposed method, when used for estimation of curcumin after spiking with 50, 100 and 150% of additional drug, afforded recovery ranging from 98.35% - 100.68% for curcumin was obtained as listed in (Table 2).The RSD of recovery of curcumin was ranged from 0.10 -0.37 (Table 2).

Table 2

Recovery study of curcumin.

Excess drug added to the analyte (%)	Theoretical content(ng)	%Recovery	%RSD
0	100	98.35	0.10
50	150	99.45	0.37
100	200	99.49	0.34
150	250	100.68	0.18

RSD = Regressed standard deviation (RSD, %) = standard deviation /mean× 100

Table 3

Intra and inter day precesion of HPTLC method (n=6) for curcumin.

Nominal concentration /ng per spot	Concentration founda/ng per spot	Precisionb (CV, %)	Accuracyc, %
Intra-day			
200	198.65	1.63	99.32
400	396.85	1.20	99.21
800	806.12	1.13	100.71
Inter-day			
200	197.11	2.25	98.55
400	394.86	1.33	98.71
800	795.45	1.52	99.43

a. Mean of six determinations (n = 6).
b. Precision as coefficient of variation (CV, %) = standard deviation divided by concentration found x100.
c. Accuracy = concentration found/nominal concentration ×100.

Table 4

Robustness of the method (n=3) for curcumin.

Curcumin	Mobile phase composition	
Amount (ng/spot)	Tolouene: Chloroform: Methanol (5:4.5:0.5v/v/v) % RSD	Toluene: Chloroform: Methanol (5:4:1 v/v/v) % RSD
200	1.34	0.80
400	0.85	1.86

3.3.2. Precision and accuracy

The intra- and inter-day precision, as coefficient of variation (CV, %) and accuracy of the assay determined at curcumin concentration of 200, 400 and 800 ng/spot has been

Table 5

Curcumin content in sample extract (% w/w of sample).

S.No.	Accession No.	Cultivation region	Geographical region	(% w/w) of sample Mean ± SD
1	32	Guwahati (ASSAM)	North -east	1.309± 0.003
2.	109	Nasik (MAHARASTRA)	North-west	0.991±0.005
3.	5	Patna(BIHAR)	East	1.62 ± 0.05
4	77	Delhi (DELHI)	North	0.882±0.006
5	9	Trivendrum (KERALA)	South	1.563 ±0.086
6	451	Lukhnow(UTTARPRADESH)	North-central	0.730± 0.026
7	21	Surat(GUJRAT)	West	0.516± 0.061
8	67	Erode (TAMILNADU)	South	1.82 ± 0.030

summarized in (Table 3). The intra-day precision (n = 6) was ≤1.63%. The inter-day precision over three different days was ≤2.25%. The intra-day and inter-day accuracy were in the range of 99.32% – 100.71% and 98.55% – 99.43%, respectively. The repeatability of the method was studied by assaying six samples of curcumin at same concentration under the same experimental conditions. The values were within the acceptable range and so we concluded that the method was accurate, reliable and reproducible (Table 3).

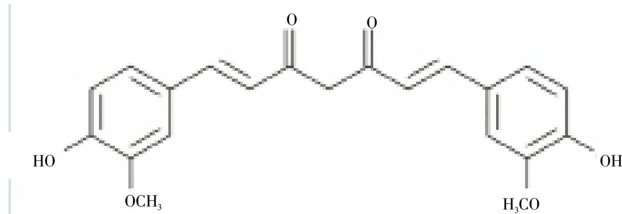

Figure 1. Chemical structure of curcumin (diferuloylmethane).

3.3.3. Robustness of the method

The standard deviation of peak areas was calculated for each parameter and RSD was found to be in the acceptable range .The low values of SD (<3.0) and % RSD (<1.2) obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness of the method (Table 4).

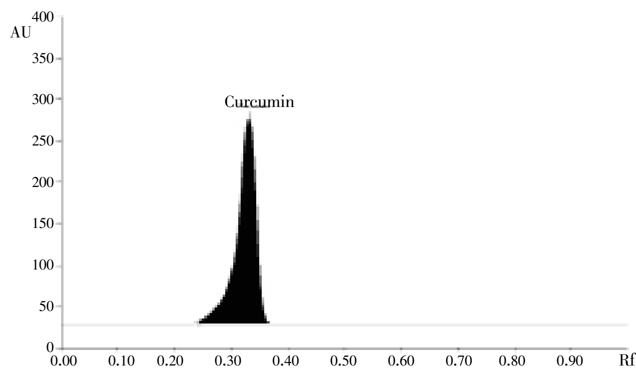

Figure 2. TLC Chromatogram of curcumin (RF= 0.31± 0.02)

3.3.4. LOD and LOQ

The calibration curve in this study was plotted between amount of analyte versus average response (peak area) and the regression equation was obtained Y = 6597.142+ 13.240 X over the concentration range 200–1000 ng/spot with respect to the peak area with a regression coefficient of 0.99354. Limit of detection and limit of quantification was calculated by the method as described in validation section and was found to be 50 and 200 ng respectively, which indicates the ample sensitivity of the method.

3.3.5. Specificity

The specificity of the proposed method was determined by comparing the sample and standard peak for its RF and UV spectra. Three point peak purity i.e. peak start, peak apex, and peak end was compared and found superimposed. This indicated that standard curcumin and sample peaks were not merging with any other components or impurities. The peak purity of curcumin was assessed by comparing the spectra at three different levels, i.e. Peak start, peak apex and peak end positions (Figure 3).

A good resolved single spot of curcumin was observed at RF value 0.31±0.02 in the chromatogram of the samples extracted from rhizomes of *C. longa* collected from different regions of India. The curcumin content in different sample of *C. longa* was observed and calculated (Table 5).

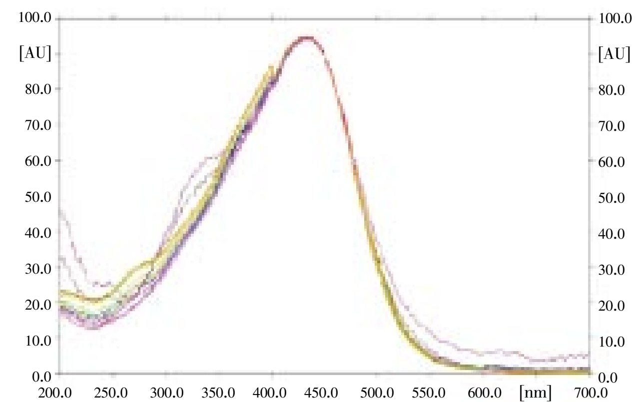

Figure 3. Superimposed spectra of Curcumin from standard and sample zones.

4. Discussion

A validated HPTLC method has been developed for the determination of curcumin in *C. longa* collected from different geographical region of India. The proposed method is simple, precise, specific, accurate, less time consuming and cost effective. Statistical analysis proved that the method is evitable for the analysis of curcumin. The developed HPTLC method will help the manufacturer for quality control and standardization of herbal formulations. Such finger printing is useful in differentiating the species from the adulterant and act as a biochemical marker for this medicinally important plant in the pharmaceutical industry[30]. In this experiment highest and lowest percentage of curcumin present in *C. longa* was found to be present in Erode (Tamilnadu, South region) and Surat (Gujrat, west region) sample respectively. The method established in this study could be used for the quality control of herbal medicines derived from Curcuma species.Experimental result shows that the variety of *C. longa* of Erode (Tamilnadu) sample is superior to other regions of India. Therefore, Erode (Tamilnadu, South region) Province could be considered as the desirable cultivation region for the production *C. longa*.

Conflict of interest statement

We declare that we have no conflict of interest.

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